

## Protective Effects of Pertussis Immunoglobulin (P-IGIV) in the Aerosol Challenge Model

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**Pertussis in infants is often severe, resulting in prolonged hospitalization. Treatment is limited to supportive care. Antibiotics do not significantly alter the course of the disease unless administered during the catarrhal phase. Therapies directed at pertussis toxin, a major virulence factor of *Bordetella pertussis*, may be beneficial. This study uses the aerosol challenge model to further examine the protective effects of P-IGIV, a new intravenous immunoglobulin product, which has high levels of pertussis toxin antibodies. P-IGIV was prepared as a 4% immunoglobulin G (IgG) solution from the pooled donor plasma from donors immunized with inactivated pertussis toxoid. The IgG pertussis toxin antibody concentration in P-IGIV is >7-fold higher than conventional intravenous immunoglobulin products. In the aerosol challenge model, P-IGIV-treated mice exhibited a dose-dependent decrease in mortality when monitored for 28 days postchallenge. P-IGIV in doses of 2,800, 1,400, and 350 mg/kg significantly reduced mortality compared to saline ( $P < 0.01$ ) and human IGIV ( $P < 0.01$ )-treated controls. The 50% protective dose of pertussis toxin antibodies in P-IGIV was 147  $\mu$ g/ml. Recovery of weight gain and normalization of leukocyte counts occurred in all P-IGIV-treated groups but did not exhibit dose-dependent characteristics. Even after 7 days of infection, P-IGIV reversed the effects of pertussis in mice. This study provides further evidence that pertussis toxin antibodies not only play a role in passive protection but can also reverse symptoms of established disease in mice. We feel that P-IGIV deserves further evaluation in children hospitalized with severe pertussis.**

Pertussis continues to cause significant morbidity and mortality in young infants and children throughout the world, even in well-immunized populations (13–17, 32). The disease is characterized by severe paroxysmal coughing and choking in infants and whooping in older patients. Pertussis can be severe and even fatal in unimmunized children. Although erythromycin is effective in eliminating *Bordetella pertussis* from the nasopharynx of infected patients, it does not substantially alter the course of the illness unless it is initiated during the catarrhal phase (7, 41). Additional therapies that decrease the duration and severity of pertussis are needed.

Pittman hypothesized that the systemic manifestations of pertussis are mediated by pertussis toxin (PT); however, the mechanism by which PT might cause paroxysmal coughing has not been elucidated (42). Recent clinical vaccine trials have demonstrated that acellular pertussis vaccines confer protection against pertussis (1, 2, 9, 19, 24, 27, 40, 60, 62). Although many of these vaccines contain secondary antigens, there is evidence that antibodies to PT alone are effective in protecting against severe pertussis, as shown in single-component pertussis toxoid vaccine trials (1, 62). Despite demonstrated efficacy of the vaccines, laboratory measurement of antibodies has not demonstrated a level that corresponds to protection (35, 61). There is still no known therapy for established disease in humans and no therapy directed specifically at PT.

In the past pertussis immunoglobulin preparations made from pooled convalescent sera were investigated in trials that resulted in inconclusive efficacy data (5, 10, 29, 33, 34, 38, 58). In a recent Swedish study, a high-titer pertussis immunoglobulin significantly decreased whooping in hospitalized patients with pertussis (23). Because of the need for further investiga-

tion into the use of anti-PT antibodies as therapy for established pertussis, the Massachusetts Public Health Biologic Laboratories (MPHBL) developed a high-titer antipertussis immunoglobulin (P-IGIV) for intravenous administration in humans.

In this study we evaluated the therapeutic effect of P-IGIV on established pertussis by utilizing the aerosol challenge model as first described by Sato et al. (56, 57). We studied the pharmacokinetics of P-IGIV and, specifically, of human anti-PT immunoglobulin G (IgG) antibodies in murine systems. We have also studied the therapeutic potential of P-IGIV by measuring the effect of P-IGIV on leukocytosis, weight gain, and mortality in young mice with established pertussis. We have examined the effect of dose and timing of P-IGIV on established disease. We provide further evidence that pertussis toxin antibodies play a significant role in the treatment of and recovery from established disease.

### MATERIALS AND METHODS

**Mice.** Female specific-pathogen-free BALB/c mice with natural litters were ordered from Charles River Laboratories and timed to arrive 4 days after dropping their litters. Mice were housed one litter per cage prior to weaning, and five mice per cage after weaning, in autoclaved Low Profile Micro-Isolator (Lab Products, Inc., Maywood, N.J.) filtered top cages with standard bedding. Mice had free access to autoclaved food and water. The cages of mice were placed in a portable HEPA-filtered Ventilated Animal Rack in the BL-2 containment suite. All procedures and handling of mice took place in a BiochemGARD hood. All mice were marked by using standard ear-clipping methods. Blood samples were obtained by retro-orbital bleeding (50 to 100  $\mu$ l) of appropriately anesthetized animals as described previously (8).

**Bacteria.** *Bordetella pertussis* 18323 (ATCC 9797; American Type Culture Collection, Rockville, Md.) was recovered from a lyophilized stock from the MPHBL and inoculated onto Bordet-Gengou (BG) agar (Difco Laboratories, Detroit, Mich.) with 15% defibrinated horse blood. Growth from 72-h cultures was transferred to fresh BG plates containing 15% defibrinated horse blood and grown at 35°C for 21 h. Bacteria were then removed from the plate by using a sterile loop and resuspended in phosphate-buffered saline (pH 7.4) for use in aerosolization.

The concentration of bacteria in the nebulized solution was set to deliver approximately  $10^9$  organisms per ml for 30 min, an optimal concentration as

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TABLE 1. Comparison of P-IGIV to other globulin preparations

Globulin preparation	Antitoxin <sup>a</sup> level (titer <sup>-1</sup> )	Anti-PT IgG <sup>b</sup> concn (μg/ml)
Hyperimmune globulins		
P-IGIV (lot MIA-A164)	2,048	171
Cutter "old" PIG	88	3.6
Merieux PIG (Sweden)	640	ND
Standard immunoglobulins		
Cutter Gamimmune N	29	1.71
Sandoz Sandoglobulin	15	0.93
Immuno AG	13	0.54
MPHBL immunoglobulin 16.5% (HuISG)	ND <sup>c</sup>	21.8
Murine hyperimmune serum: PHIS	32	37.9

<sup>a</sup> Determined by CHO cell assay.<sup>b</sup> Determined by ELISA.<sup>c</sup> ND, assay not done.

demonstrated by other investigators (39, 54). Final concentrations were determined by spectrophotometry and confirmed by subsequent culturing of serial dilutions on BG agar. In one of the timing experiments the nebulized concentration was approximately  $10^{10}$  CFU/ml, resulting in slightly higher mortality, but the results were consistent with other experiments, and so the results were combined and analyzed.

**Globulin preparations.** P-IGIV was prepared as a 4% IgG solution by cold ethanol fractionation from the pooled plasma of donors who had been immunized with 50 μg of tetranitromethane (TNM)-inactivated pertussis toxoid (PTx). P-IGIV was lyophilized in vials, stored at 4°C, and reconstituted with sterile water to achieve the desired concentration of 10% globulin. Serial dilutions of the 10% solution were used to achieve lower concentrations of P-IGIV. The 10% solution was enriched >40-fold for anti-PT antibodies relative to conventional 5% IGIV and previously licensed intramuscular pertussis immunoglobulin products (20–22, 44) as seen in Table 1.

Mouse polyclonal hyperimmune serum (PHIS) was a control mouse serum made from pooled polyclonal sera of mice immunized with TNM-inactivated PTx vaccine. Human standard gammaglobulin (HSG) for intramuscular use (MPHBL lot ISG-98) was obtained from the Massachusetts Public Health Biologic Laboratories and was used as a control human gamma globulin in the pharmacology experiments, and Cutter Gamimmune 5% globulin was used as a control for the aerosol challenge experiments. A comparison of the globulin and anti-PT IgG concentrations of the globulin preparations can be found in Table 3.

**Serologic methods.** (i) **Human anti-PT IgG assays.** Mouse sera were assayed for detection of human anti-PT antibody concentrations by methods modified from those previously described by Siber et al. (59). Briefly, 96-well plates (Dynatech Immulon 2; Dynatech, Alexandria, Va.) were coated with purified PT (1.0 μg/ml) obtained from the MPHBL. Sensitized plates were incubated overnight at 4°C with serial twofold dilutions of a known A70 human antibody standard or serial fourfold dilutions of the unknown test sera (five dilutions). Bound antibodies were detected by using goat anti-mouse IgG human-absorbed alkaline phosphatase-conjugated antibodies (Caltag Laboratories, San Francisco, Calif.).

The anti-PT IgG antibody concentrations for the A70 standard were determined by the Zollinger method as previously described (59, 63). By using the Center for Biologics Evaluation and Review (CBER) pertussis reference antiserum (lot 3), 1 μg/ml was determined to be equivalent to 10 CBER U/ml for PT IgG.

(ii) **Mouse anti-PT IgG assays.** Similar methods were used to detect mouse anti-PT IgG antibodies. Briefly, 96-well plates (Dynatech Immulon 2) were coated with purified PT (1.0 μg/ml) obtained from the MPHBL. Sensitized plates were incubated overnight at 4°C with serial twofold dilutions of PHIS obtained from the MPHBL (10 dilutions), or serial fourfold dilutions of the unknown test sera (five dilutions). Bound antibodies were detected by using goat anti-mouse IgG human-absorbed alkaline phosphatase-conjugated antibodies (Caltag Laboratories, San Francisco, Calif.). The anti-PT IgG antibody concentrations for the A70 standard were determined by the Zollinger method as previously described (59, 63).

**Pharmacokinetic analysis.** The pharmacokinetics of P-IGIV were studied by a comparison of class- and antigen-specific antibodies. The mean volume of distribution (*V*) and the half-life (*t*<sub>1/2</sub>) of anti-PT antibodies were calculated to compare the pharmacokinetics of PT-specific antibodies after intravenously (i.v.) and intramuscularly administered P-IGIV in mice. The half-life calculations were determined in uninfected mice. *t*<sub>1/2α</sub> was not calculated, while *t*<sub>1/2β</sub> was calculated by using the terminal elimination phase (3 to 90 days). The volume of distribution and half-life calculations are reported as geometric means. The 50% pro-

tective dose (PD<sub>50</sub>) for P-IGIV was estimated by the Reed and Muench method as previously described (46).

**WBC concentrations.** Leukocyte (WBC) concentrations were measured by obtaining 20 μl of blood by retro-orbital bleeding by using the Unopette capillary tube system (Becton Dickinson, Rutherford, N.J.). Anesthetized mice were bled at specified times post-aerosol challenge. WBC counts were calculated as thousands of cells per cubic millimeter by using a hemocytometer and ×40 microscope.

**Statistical methods.** Data organization and analysis were performed with the PROPHET system, a national computer system sponsored by the National Center for Research Resources of the National Institutes of Health. For all antibody measurements, geometric means were calculated. Values below the lower limit of sensitivity of the assay were assigned a value of half the lower limit for purposes of taking logarithms. Comparisons of means or geometric means were performed by the two-sided *t* test for normally distributed values and by the Mann-Whitney test for nonnormally distributed values. Comparisons of proportions were performed by a two-sided Fisher exact test.

**Aerosol challenge.** Mice were removed from their cages, weighed, and placed on a stainless steel rack that fits inside of a Plexiglas aerosol chamber (40 by 40 by 40 cm). The 21-h culture of *B. pertussis* was resuspended in sterile phosphate-buffered saline to a concentration of approximately  $10^9$  CFU of inoculum per ml. This inoculum was delivered to the mice by using a standard nebulizer (Divilbis model 647 nebulizer; Divilbis, Summerset, Pa.) with a set pressure of 1.5 kg/cm<sup>2</sup>. The chamber and the nebulizer were enclosed in the BL-2 biosafety hood and certified prior to use to document that airflow barriers were maintained. Uniformity of the aerosol in the chamber was maintained with the use of two PAPST 900 series AC fans (Newark Supply, Newark, N.J.) and monitored by a laser light source to confirm an even dispersion of the aerosol droplets. Mice were exposed to nebulization for 30 min and removed 30 min after termination of aerosolization. The completion of the aerosol represented time zero. Mice were removed from the box and returned to their cages. Cages were checked daily for mortality.

**Establishment of the model.** Experiments were designed to examine the pharmacokinetics, dose response, and effect of timing of P-IGIV in the aerosol challenge model. Because P-IGIV is developed for i.v. administration and because intraperitoneal (i.p.) administration is immensely easier in neonatal mice, the basic pharmacology of P-IGIV was studied in uninfected mice, comparing i.v. administration to i.p. administration. We also compared P-IGIV to mouse PHIS and a standard human gamma globulin (HuISG) preparation. Twenty-four-day-old mice were used to approximate the age of mice in the aerosol challenge experiments (17 days old plus 7 days postchallenge). Groups of five mice received P-IGIV at a dose of 1,700 mg/kg in 0.2 ml given either i.p. or i.v. Mice were bled at 1, 3, and 6 h and at 3, 7, and 14 days after administration. Serum half-life and volume of distribution calculations were determined as described above.

In order to study the dose response of P-IGIV, pilot experiments were done to determine the optimal timing for P-IGIV treatment doses. Mice were treated with a single i.p. dose of P-IGIV at various times after aerosol challenge. Using groups of 10 mice, treatment with P-IGIV in a dose of 1,000 mg/kg resulted in the survival of 60% with treatment given on day 6, 10% with treatment given on day 8, and 0% with treatment given on day 9 postchallenge. Mortality in 18-day-old untreated mice was consistently 100% by 14 days postchallenge. For subsequent experiments comparing P-IGIV doses, 7 days postchallenge was set as the optimal time for treatment. Of 151 mice, 11 died before treatment was given 7 days after challenge. At 7 days mice were treated with P-IGIV in doses of 2800, 1400, 700, 350, 175, and 88 mg/kg; with human IGIV (Gamimmune N 4%), 2,800 mg/kg; or with saline.

We also studied the effect of timing of P-IGIV. For all timing experiments a P-IGIV dose of 1,000 mg/kg was used. We combined the data of two experiments which examined different timing schedules for P-IGIV administration. In the first experiment mice were challenged with *B. pertussis* and then treated with P-IGIV on day 6, 10, 12, 14, or 18 postchallenge. In a second experiment, mice were challenged with *B. pertussis* and then treated with P-IGIV on day 6, 7, 8, 9, or 10 postchallenge. The results of these two experiments were combined for the timing data presented below.

## RESULTS

**Pharmacology of P-IGIV.** The geometric mean anti-PT IgG antibody level measured 1 h after the i.v. dose was 89.3 μg/ml, and that measured 3 h after the i.p. dose was 38.1 μg/ml. These concentrations were not statistically different (Table 2 and Fig. 1). The levels continued to be similar thereafter for i.v. and i.p. dosages. The geometric mean volumes of distribution (*V*) based on the antibody concentrations at 3 days were 4.26 ml after i.v. administration and 3.53 ml after i.p. administration (*P* > 0.05). The *t*<sub>1/2</sub> values of the geometric mean anti-PT IgG antibody concentrations for P-IGIV were 7.8 days after i.v. and 7.0 days after i.p. administration (*P* > 0.05).

We compared the pharmacology of P-IGIV given i.p. with a

TABLE 2. Pharmacokinetics of mouse and human anti-PT IgG antibodies administered both i.v. and i.p. in BALB/c mice<sup>a</sup>

Preparation	Anti-PT IgG dose (mg/kg)	Observed peak (h)	Peak level <sup>b</sup> (μg/ml)	V (ml)	t <sub>1/2</sub> (days)
P-IGIV (i.v.)	146.6	1	89.3	4.26	7.8
P-IGIV (i.p.)	146.6	3	38.1	3.53	7.0
PHIS (i.p.)	181.9	3	101.8	2.00	5.8
HuISG (i.p.)	4.4	6	2.14	2.33	6.9

<sup>a</sup> All values reported are geometric means.<sup>b</sup> The peak P-IGIV administered i.p. was significantly higher than the peak HuISG ( $P < 0.05$ ).

control human immunoglobulin (HuISG) given in a dose of 2,800 mg/kg in 0.2 ml i.p. The geometric mean peak concentration of anti-PT IgG antibody of 2.14 μg/ml observed 6 h after HuISG administration was significantly lower than that with i.p.-administered P-IGIV ( $P = 0.024$ ). The geometric mean  $V$  of HuISG based on levels at 3 days was 2.33 ml, and the  $t_{1/2}$  was 6.9 days.

In order to compare the clearance of human and murine IgG in the model, we measured murine anti-PT IgG antibodies after i.p. administration of PHIS in a 0.2-ml volume. The peak geometric mean level of anti-PT IgG antibodies of 101.8 μg/ml was reached in 3 h. The geometric mean  $V$  based on levels at 3 days was 2.00 ml, and the  $t_{1/2}$  was 5.8 days.

**Dose response of P-IGIV.** Saline-treated controls showed 50% mortality by day 11 and 100% mortality by day 14. The Gamimmune-treated control mice showed 50% mortality by day 13 and 100% mortality by day 17. The P-IGIV-treated mice exhibited a dose-dependent decrease in mortality, monitored for 28 days postchallenge (Tables 3 and 4). P-IGIV in doses of 2,800, 1,400, and 350 mg/kg all significantly reduced mortality compared to saline ( $P < 0.01$ )- and Gamimmune ( $P < 0.01$ )-treated controls. P-IGIV in a dose of 700 mg/kg also reduced mortality compared to saline and Gamimmune but because of one outlier did not achieve statistical significance ( $P > 0.05$ ). Even after established infection for 7 days, P-IGIV reversed the mortality due to pertussis in mice in the aerosol challenge model, exhibiting dose-dependent characteristics (Table 4). Using the Reed and Muench estimation method, the  $PD_{50}$  for P-IGIV was found to be approximately 0.8% globulin, which corresponds to approximately 147 μg of anti-PT IgG antibodies per ml.

By 14 days after challenge all mice treated with saline and Gamimmune showed a marked leukocytosis and weight loss

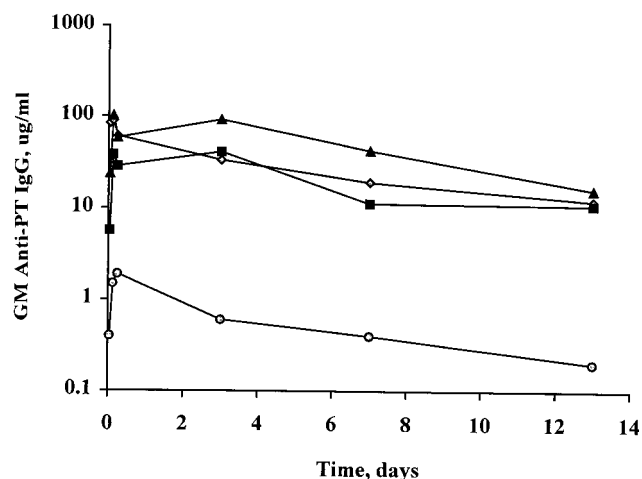


FIG. 1. Pharmacokinetics of human and mouse anti-PT IgG antibodies in BALB/c mice. There was no significant difference ( $P > 0.05$ ) when the values for volume of distribution and half-life for i.v. administered P-IGIV and i.p. administered P-IGIV were compared. The clearance of mouse anti-PT IgG antibodies was similar to the clearance of human anti-PT IgG antibodies in BALB/c mice. GM, geometric mean. Symbols: ◇, P-IGIV (i.v.); ■, P-IGIV (i.p.); ▲, PHIS (i.p.); and ○, HuISG (i.p.).

prior to death. Recovery of weight gain and normalization of WBC counts occurred in all P-IGIV-treated groups but did not exhibit dose-dependent characteristics (Table 5). Mean WBC counts at day 7 postchallenge (pretreatment) were not statistically different. Although there was a significant rise in the WBC count prior to treatment, all P-IGIV-treated groups showed normalization of leukocytosis by 28 days postchallenge; that is, the WBC counts were no longer statistically different from those of uninfected controls ( $P > 0.05$ ). Mean weights were statistically similar for all groups on the day of challenge. All P-IGIV-treated groups were smaller than uninfected controls on day 14 postchallenge ( $P < 0.01$ ) but had recovered their weight by day 28 and were no longer significantly different ( $P > 0.05$ ). In fact, the survivors in all P-IGIV-treated groups exhibited accelerated weight gain in order to reach the normal weight curve of the uninfected controls (Table 5). The saline-treated controls all lost a significant amount of weight compared to the uninfected controls ( $P < 0.01$ ) prior to their death. Even after established infection for 5 to 7 days, P-IGIV reversed the severe effects of pertussis such as leuko-

TABLE 3. Comparison of anti-PT IgG antibody concentration in P-IGIV doses and with standard IGIV<sup>a</sup>

IgG antibody (dose [mg/kg])	Globulin concn (%)	Approximate globulin dose (mg/kg)	Anti-PT IgG concn (μg/ml)	Anti-PT IgG dose (mg/kg)
P-IGIV (2,800)	10	2,800	1,832.5	50.97
P-IGIV (1,400)	5	1,400	916.3	25.49
P-IGIV (700)	2.5	700	450	12.74
P-IGIV (350)	1.25	350	225	6.37
P-IGIV (175)	0.625	175	112	3.19
P-IGIV (88)	0.3125	88	56	1.59
Gamimmune	4	1,100	5.3	0.15

<sup>a</sup> All doses of P-IGIV had significantly higher anti-PT IgG levels than did saline ( $P < 0.01$ ) and Gamimmune ( $P < 0.05$ ), except for 700-mg/kg P-IGIV ( $P > 0.05$ ). Globulin and anti-PT IgG doses are based on the mean weight at the day of challenge for all groups (7.2 g) and an administered volume of 0.2 ml.

TABLE 4. Effect of P-IGIV dose on survival after aerosol challenge with *B. pertussis*<sup>a</sup>

Group (dose [mg/kg])	50% Mortality (days postchallenge)	% Survival postchallenge at:			
		7 days	14 days	21 days	28 days
Uninfected controls	>28	100.0	100.0	100.0	100.0
P-IGIV (2,800)	>28	96.7	96.7	93.3	90.0
P-IGIV (1,400)	>28	96.7	90.0	90.0	90.0
P-IGIV (700)	>28	97.1	91.4	88.6	85.7
P-IGIV (350)	>28	93.3	83.3	83.3	83.3
P-IGIV (175)	21	85.7	51.4	42.9	42.9
P-IGIV (88)	21	100.0	60.0	40.0	40.0
Gamimmune	13	100.0	45.5	0.0	0.0
Saline	11	78.9	0.0	0.0	0.0

<sup>a</sup> All treatment doses were administered 7 days after aerosol challenge. P-IGIV doses of 2,800, 1,400, and 350 mg/kg significantly reduced mortality compared to the saline ( $P < 0.01$ ) and Gamimmune ( $P < 0.01$ ) groups.



TABLE 5. Effects of P-IGIV dose on leukocytosis and weight loss after aerosol challenge in mice

Group (dose [mg/kg])	Geometric mean WBC counts (10 <sup>3</sup> cells/mm <sup>3</sup> ) at (days postchallenge):					Change in wt (g) at (days postchallenge) <sup>g</sup> :			
	3 days <sup>a</sup>	7 days <sup>b</sup>	14 days	21 days	28 days <sup>c</sup>	0-7 days <sup>d</sup>	7-14 days	0-14 days	0-28 days <sup>e</sup>
P-IGIV									
2,800	30.3	70.2	34.7	29.6	5.1	-0.3	2.2	1.9	9.5
1,400	38.5	76.0	15.7	32.7	7.4	-0.3	2.9	2.6	9.0
700	32.1	92.8	25.7	14.2	8.5	-0.6	1.8	1.2	9.6
350	26.1	54.4	12.9	8.6	6.0	0.7	3.4	4.1	10.3
175	23.7	71.0	75.8	8.3	5.8	-0.1	2.9	2.9	9.9
88	20.0	85.3	83.5	12.5	6.0	0.5	2.4	2.9	10.0
Gamimmune	28.0	130.2	194.7			0.4	—		
Saline	48.9	175.7	— <sup>f</sup>			-0.5	—		
Uninfected controls	7.7	6.0	9.3	8.7	7.1	3.7	3.6	7.3	9.2

<sup>a</sup> There was no difference between all P-IGIV groups and the saline group ( $P > 0.05$ ), except for the 88-mg/kg P-IGIV group ( $P < 0.05$ ).

<sup>b</sup> All P-IGIV values were significantly higher than the uninfected controls ( $P < 0.01$ ), except for the 175-mg/kg P-IGIV group ( $P > 0.05$ ).

<sup>c</sup> All P-IGIV groups had WBC counts similar to those of uninfected controls ( $P > 0.05$ ).

<sup>d</sup> On day 14, all P-IGIV groups weighed significantly less than the uninfected controls ( $P < 0.01$ ).

<sup>e</sup> By day 28 all P-IGIV groups had regained normal weight ( $P > 0.05$ ).

<sup>f</sup> —, all mice were dead prior to day 14.

<sup>g</sup> Change in geometric mean.

cytosis and weight loss in mice in the aerosol challenge model but, unlike mortality, these effects did not exhibit dose-dependent characteristics.

**Effects of timing.** Saline-treated controls showed 50% mortality by day 12 and 100% mortality by day 13. Mice treated with P-IGIV on day 6 or 7 post-aerosol challenge had greater than 50% survival at day 28 (Table 6). Although there appeared to be a trend toward reducing mortality with earlier treatment, only when P-IGIV was administered on day 6 or 7 postinfection did it achieve a statistically significant improvement over saline ( $P < 0.01$ ) (Table 6).

By 6 days after challenge all mice treated with saline showed a marked leukocytosis and weight loss prior to death. Recovery of weight gain and normalization of WBC counts occurred in all P-IGIV-treated groups but did not exhibit dose-dependent characteristics (Table 7). Mean WBC counts at day 3 postchallenge (pretreatment) were not statistically different for any of the groups infected with *B. pertussis*. Although there was a significant rise in WBC count prior to treatment ( $P < 0.05$ ), all P-IGIV-treated groups showed normalization of leukocytosis by 28 days postchallenge and were no longer statistically different from uninfected controls ( $P > 0.05$ ). Mean weights were statistically similar for all groups on the day of challenge. All P-IGIV-treated animals were significantly smaller than uninfected controls on day 14 postchallenge ( $P < 0.01$ ) but had recovered their weight by day 28 ( $P > 0.05$  versus uninfected

controls). The saline-treated controls all lost a significant amount of weight prior to their deaths.

## DISCUSSION

There is compelling evidence to date that PT antibodies alone provide passive protection against pertussis. Although protective levels of PT antibodies have not been determined, vaccine trials and specifically those using single component acellular PT vaccines have shown that antibodies against PT alone can protect against pertussis. The addition of other antigens such as filamentous hemagglutinin (FHA), agglutinins, and 69-kDa protein only add minimal protection to that achieved by PTx alone (1, 9, 62). It is known that patients convalescing from pertussis mount a high antibody response to PT, which approximates the onset of gradual recovery from the disease (40, 41).

It is believed that PT may be responsible for the characteristic cough of pertussis whether locally or centrally mediated. The therapeutic role of anti-PT antibodies in patients with established pertussis was not conclusively demonstrated in the past. Pertussis immunoglobulin preparations made from pooled convalescent sera were investigated in trials that resulted in inconclusive efficacy data (5, 10, 29, 33, 34, 38, 58). Subsequent testing has shown that some of these products had low levels of anti-PT IgG antibodies, as seen in Table 1. By using a pertussis immunoglobulin made at Institute Merieux, Granström et al. demonstrated that PT antibodies significantly reduced whooping in children (23). P-IGIV was developed as an i.v. product to further evaluate the role of PT antibodies in the treatment of pertussis. Two important differences in P-IGIV should be mentioned. The Swedish product was given intramuscularly, which results in lower peak levels of PT antibodies, and their product had lower titers of PT antibodies as measured by enzyme-linked immunosorbent assay (ELISA) and CHO cell assay. Because P-IGIV has a high titer of anti-PT antibody it has promise as a therapeutic agent, especially when given i.v.

There are several animal models in which antibodies to PT have been shown to be protective, such as histamine challenge, intracerebral inoculation, and intranasal challenge (18, 26, 28, 43, 47). We chose the aerosol challenge model as described by Sato et al. (56) to further study P-IGIV since it has many

TABLE 6. Effect of timing of P-IGIV on mortality after aerosol challenge in mice<sup>a</sup>

Group	50% Mortality (days postchallenge)	% Survival postchallenge at:			
		7 days	14 days	21 days	28 days
Uninfected	>28	100.0	100.0	100.0	100.0
P-IGIV					
Days 6 and 7	>28	86.7	56.7	53.3	53.3
Days 8 and 9	11	78.9	5.3	5.3	5.3
Days 10 and 12	12	100.0	3.3	3.3	3.3
Days 14 and 18	13	100.0	0.0	0.0	0.0
Saline	12	92.0	0.0	0.0	0.0

<sup>a</sup> All mice received P-IGIV (1,000 mg/kg). Only P-IGIV administered 6 days after aerosol challenge significantly improved survival compared to the saline group ( $P < 0.01$ ).

TABLE 7. Effects of timing of P-IGIV on leukocytosis and weight loss after aerosol challenge in mice

Group	Geometric mean WBC count ( $10^3$ cells/mm <sup>3</sup> ) at (days postchallenge):				Change in wt (g) at (days postchallenge) <sup>b</sup> :			
	3 days	6 days	10 days	14 days	0-7 days	7-14 days	0-14 days	0-28 days
Uninfected controls	5.9	7.0	7.3	10.2	3.7	3.9	7.6	9.6
P-IGIV <sup>a</sup>								
Days 6 and 7	28.5	104.1	70.5	45.7	0.5	2.5	3.1	8.9
Days 8 and 9	29.1	157.9	219.6	68.5	-0.1	-0.04	-0.1	3.6
Days 10 and 12	23.2	111.3	259.3	237.5	1.6	-1.7	-0.1	
Days 14 and 18	24.2	108.9	263.4		1.9	-1.8	0.2	
Saline	31.5	98.3	288.5		0.3			

<sup>a</sup> All survivors in the P-IGIV-treated groups showed normalization of leukocytosis by day 28 postchallenge, a finding not statistically different from the results for uninfected controls at day 28 ( $P > 0.05$ ).

<sup>b</sup> Change in geometric mean.

similarities to pertussis infection in infants. Although mice do not whoop or cough, the infection is established by adherence of the organisms to the columnar respiratory epithelium of mice, followed by proliferation of organisms in the lungs, lymphocytosis, weight loss, and death (56). There is evidence that illness in mice is produced by the elaboration of PT, which has been shown to cause lymphocytosis and weight loss (25). As in humans, infant mice manifest more severe illness than adult mice do (56).

Both passive and active PT antibodies have been shown to provide protection in mice when given prior to aerosol challenge with *B. pertussis* (51, 52, 54, 55, 57). Although monoclonal and polyclonal antibodies to PT provide protection when given prior to aerosol challenge (39, 51, 52, 54, 55, 57), the therapeutic benefits after established aerosol infection have not been thoroughly examined. Sato and Sato have shown that monoclonal and polyclonal antibodies to PT can reverse the severe manifestations of pertussis in mice even when administered 3 to 9 days after aerosol challenge (54).

In this study we have shown by using the aerosol challenge model that we can reverse the severe effects of established infection with *B. pertussis* in mice. Mortality demonstrated dose-dependent characteristics. The PD<sub>50</sub> was approximately 250 µg of P-IGIV (0.8% globulin) per ml. Unlike mortality, the reduction of leukocytosis and the resumption of weight gain did not exhibit dose-dependent characteristics. This would suggest that lymphocytosis and weight loss are unrelated to the amount of elaborated PT or that the amount of PT antibodies was not dilute enough to exhibit dose-dependent characteristics. This would also suggest that the mechanisms for mortality might not be related to leukocytosis or to weight loss.

It has long been believed that once PT is elaborated it becomes irreversibly bound to target tissues and therefore, once established, the disease would be unresponsive to therapy. Adler and Morse have shown that PT-induced lymphocytosis is reversible with anti-PT antibodies (3). In studies with the aerosol challenge model, a few investigators have suggested that PT antibodies could also reverse other manifestations of illness in mice, such as mortality and weight loss, even when given 3 days after established infection. We have demonstrated that P-IGIV given early was clearly more effective than a later administration; however, even when it was given 8 days after established infection, mice showed resolution of leukocytosis and reestablished weight gain.

Although it is widely accepted that immunity to pertussis is primarily dependent on a humoral response, there has been recent work suggesting that cell-mediated immunity plays an important role in protection against pertussis both in humans and in animals (4, 11, 12, 30, 31, 36, 45, 48). This cell-mediated

response has also been shown to be important in conferring immunity after active immunization with both whole-cell vaccines and acellular pertussis vaccines (36, 45, 49, 50). This cell-mediated immunity in mice infected with *B. pertussis* is characterized by the induction of a T-cell-mediated response. There is some evidence that pertussis infection and whole-cell vaccines both induce a CD4<sup>+</sup> Th1 response, whereas acellular pertussis vaccines induce a response more characteristic of CD4<sup>+</sup> Th2 (6, 45, 49, 50).

In a recent study by Mills et al. (37), it was demonstrated that cell-mediated immunity and PT-IgG antibody response play complementary roles in conferring immunity in the aerosol challenge model. They compared three whole-cell and five acellular pertussis vaccines and demonstrated a high correlation between clinical vaccine efficacy in children and *B. pertussis* clearance from the lungs of immunized mice with the aerosol challenge model. Despite this correlation, the precise mechanisms for immunity need to be further investigated. In this study we have provided further evidence for the important role of anti-PT IgG antibodies in the immune response but do not exclude the complementary role of cell-mediated immunity.

In this study we have given further evidence that PT antibodies not only play a major role in passive protection but also can reverse symptoms of established disease for at least 7 days in mice in the aerosol challenge model. This finding has implications for the treatment of children who often present 1 to 2 weeks after the onset of symptoms. P-IGIV given in comparable doses to those proposed for human trials was significantly better at reducing mortality, lowering leukocytosis, and restoring normal weight gain than saline and even standard human gamma globulin.

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